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NEW SECONDARY METABOLITES IN THE AMPHINOMID FIREWORM *HERMODICE CARUNCULATA*

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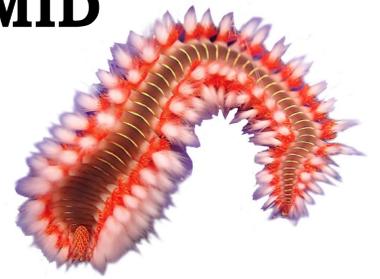
Eight betaine-derived novel compounds were found in extracts of the Mediterranean stinging fireworm *Hermodice carunculata*. The identification of their structures relies on 1D and 2D NMR (Fig. 1-3) and HPLC-ESI/HRMS spectra. Two types of terminal ammonium portions A and B and a series of different alkyl chains were identified (Fig. 4a,b). Their matching provides the structures of uncharacterized secondary metabolites, named **carunculines**, and their related isomers. These molecules differ from already known trimethylammonium inflammatory compounds (i.e. complanines) isolated from another amphinomid species, for the structures of the terminal ammonium groups (Fig. 4c) [1]. **Carunculine** anatomical distribution within *H. carunculata* was assessed by screening through HPLC-ESI/HRMS (Fig. 5, Table 1): their occurrence was revealed in all the body parts analyzed, both involved in predator-prey interactions [2], and mainly in the digestive apparatus. The results achieved reveal an array of different novel compounds from a chemically unknown species, improving knowledge on Marine Animal Products with chemical and biological potential for bioprospection [3]. Overall, these data reinforce the necessity of studying poorly-investigated taxa to expand knowledge on animal venom biology, their mechanisms of action and exploitation as promising source of drug molecules.

– Pitfalls of NMR spectra –

Our **first hypothesis**, based on ^{1}H and H,C-HSQCed spectra (Fig. 1 and 2), **was wrong**. The sign of H,C correlations in HSQCed spectrum is deceiving for cyclopropanes (^{1}J(H,C) around 160 Hz): methylenes seem methyl signals but in the COSY spectrum the intensity of the "long range" correlations between what should have been geminal CH$_3$ signals were abnormally high and with a too symmetric shape... (Fig. 3) and ^{13}C chemical shift (around 10 ppm) was too low.
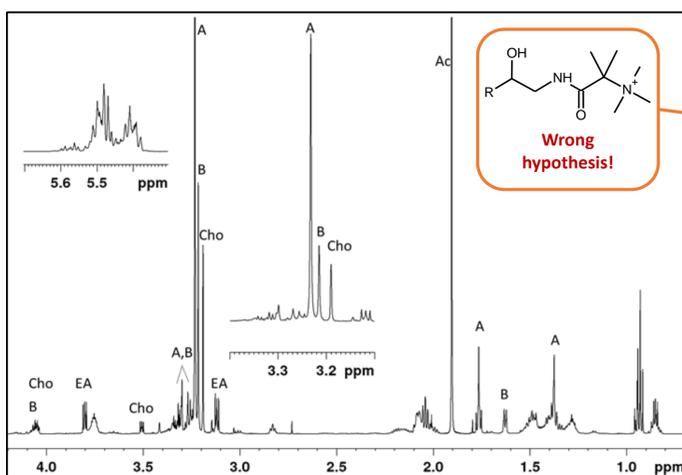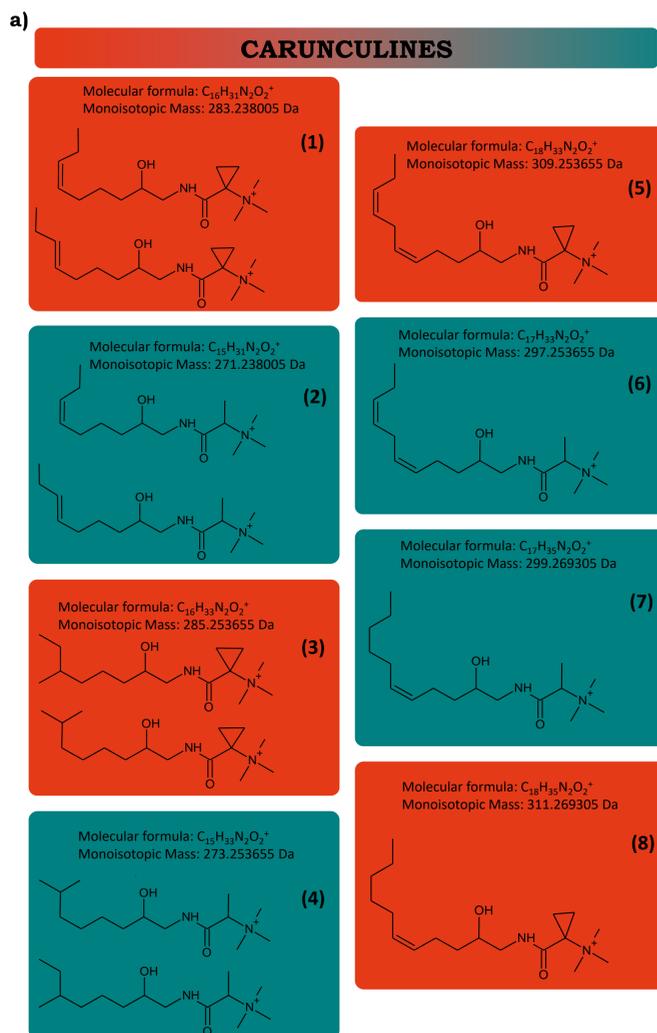

Figure 1. ^{1}H spectrum in D$_2$O of *H. carunculata* extract. Diagnostic signals of carunculines are marked with A and B. Cho = choline, EA = ethanolamine. Modified from [3].
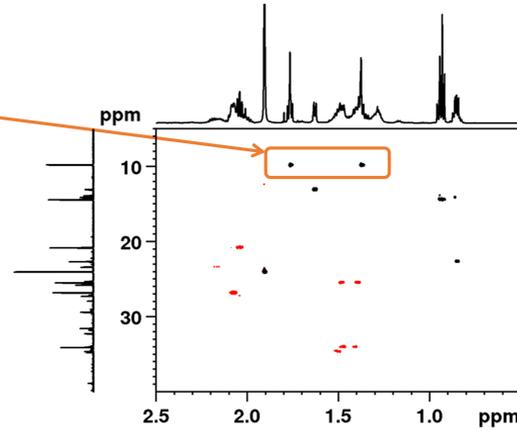
Figure 2. Enlarged regions of the H,C-HSQCed NMR spectrum. In the black rectangle, the correlation between the protons and the carbon of the cyclopropane ring, with the same sign of methyl and methyne correlations. Modified from [3].
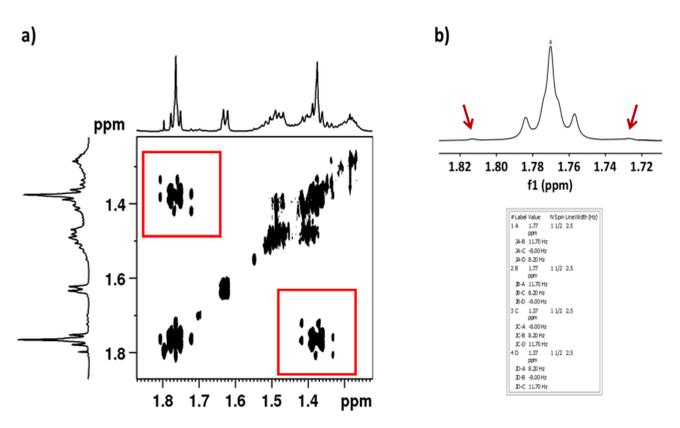
Figure 3. Enlarged region of the H,H-COSY NMR spectrum of carunculines. a) The red square shows the correlation between the AA' and BB' protons of the cyclopropane ring. b) spin-system simulation showing the shape of one of the two multiplets of the AA'BB' system (MestReNova v 14.1.2-25024). Modified from [3].

a) CARUNCULINES

Molecular formula: C$_{16}$H$_{31}$N$_2$O$_2$$^+$
Monoisotopic Mass: 283.238005 Da **(1)**

Molecular formula: C$_{18}$H$_{33}$N$_2$O$_2$$^+$
Monoisotopic Mass: 309.253655 Da **(5)**

Molecular formula: C$_{15}$H$_{31}$N$_2$O$_2$$^+$
Monoisotopic Mass: 271.238005 Da **(2)**

Molecular formula: C$_{17}$H$_{33}$N$_2$O$_3$$^+$
Monoisotopic Mass: 297.253655 Da **(6)**

Molecular formula: C$_{16}$H$_{33}$N$_2$O$_2$$^+$
Monoisotopic Mass: 285.253655 Da **(3)**

Molecular formula: C$_{17}$H$_{35}$N$_2$O$_3$$^+$
Monoisotopic Mass: 299.269305 Da **(7)**

Molecular formula: C$_{15}$H$_{33}$N$_2$O$_2$$^+$
Monoisotopic Mass: 273.253655 Da **(4)**

Molecular formula: C$_{18}$H$_{35}$N$_2$O$_2$$^+$
Monoisotopic Mass: 311.269305 Da **(8)**

Terminal ammonium portions of carunculines

Figure **4b**

A   B

Complanine   Neocomplanine A
Neocomplanine B

Figure **4c**

Figure 4. Proposed structures for carunculines and molecular structures of complanines. **a)** Proposed molecular structures for carunculines (**1–8**) and their isomers derived by matching the structures obtained by NMR spectra and the formulae obtained by HPLC-ESI/HRMS data. **b)** terminal ammonium portion (A) or (B); **c)** molecular structures of complanine and neocomplanines. Modified from [3].

RT :4.00-6.67 GNL: 2.29E9 m/z= 311.2677-311.2709 MS F: FTMS + c ESI Full ms [100.0000-1500.0000] H
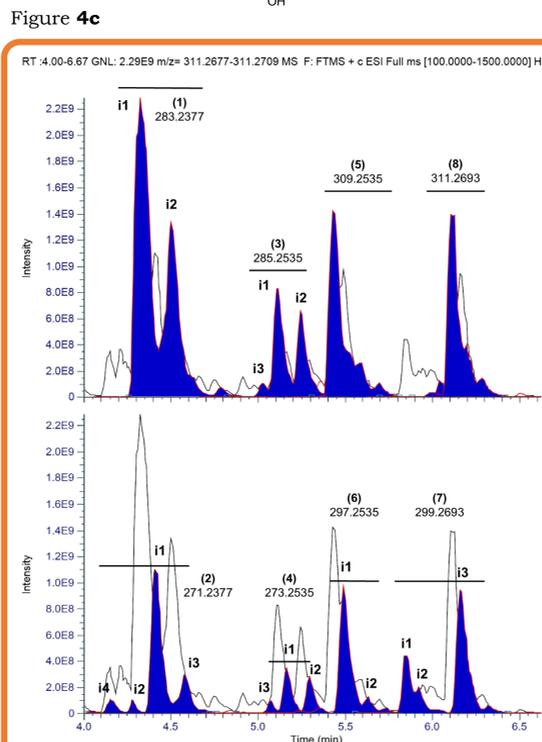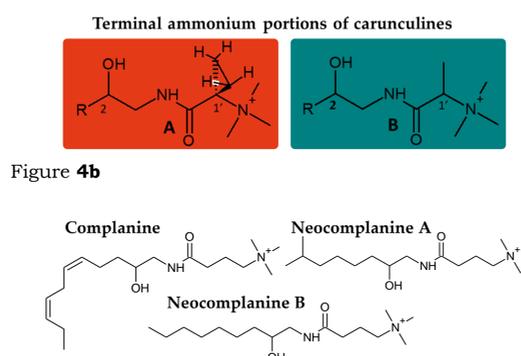
Figure 5. Extracted ion chromatogram of carunculines (**1–8**) from *H. carunculata*: the peaks of compounds **1,3,5,8** (up) and **2,4,6,7** (down) and related isomers (i1,i2,i3,i4) are filled in blue. Modified from [3].

Table 1. Relevant MS/MS fragments identified in carunculines with A (**1,3,5,8**) and B (**2,4,6,7**) using HPLC-ESI/HRMS. The fragmentation pathways are completely different, corroborating the proposed structures. Modified from [3].

Terminal ammonium portion (A)

Carunculine 1 (m/z 283.2377)	Carunculine 3 (m/z 285.2535)	Carunculine 5 (m/z 309.2535)	Carunculine 8 (m/z 311.2693)	Proposed molecular structure / formula
MS/MS fragments	MS/MS fragments	MS/MS fragments	MS/MS fragments	
58.0659	58.0659	58.0659	58.0659	CH$_2$=N(CH$_3$)$_2$$^+$
60.0815	60.0815	60.0815	60.0815	NH(CH$_3$)$_3$$^+$
67.0549	67.0549	67.0549	67.0549	C$_5$H$_7$$^+$
84.0813	84.0813	84.0813	84.0813	
95.0859	95.0859	95.0859	95.0859	C$_7$H$_{11}$$^+$
98.0967	98.0967	98.0968	98.0967	C$_6$H$_{12}$NO$^+$*
116.1071	116.1071	116.1071	116.1071	
123.1168	123.1168	/	123.1169	C$_9$H$_{15}$$^+$
143.1177	143.1178	143.1178	143.1178	H$_2$N
	neutral loss, m	*neutral loss, m*	*neutral loss, m*	*neutral loss, m*
170.1536	113.084	172.1693	113.084	196.1692	113.0843	198.1850	113.0843	
198.1850	85.053	200.2006	85.0529	224.2007	85.0528	226.2163	85.0530	
265.2270	18.011	267.2426	18.0107	291.2426	18.0109	293.2582	18.0111	– H$_2$O

*tentative structure

Terminal ammonium portion (B)

Carunculine 2 (m/z 271.2377)	Carunculine 4 (m/z 273.2535)	Carunculine 6 (m/z 297.2535)	Carunculine 7 (m/z 299.2693)	Proposed molecular structure / formula
MS/MS fragments	MS/MS fragments	MS/MS fragments	MS/MS fragments	
58.0659	58.0659	58.0659	58.0659	CH$_2$=N(CH$_3$)$_2$$^+$
60.0815	60.0815	60.0815	60.0815	NH(CH$_3$)$_3$$^+$
67.0549	67.0549	67.0549	67.0549	C$_5$H$_7$$^+$
81.0704	81.0704	81.0704	81.0704	C$_6$H$_9$$^+$
95.0859	95.0859	95.0859	95.0859	C$_7$H$_{11}$$^+$
123.1168	123.1168	123.1170	123.1169	C$_9$H$_{15}$$^+$
	neutral loss, m	*neutral loss, m*	*neutral loss, m*	*neutral loss, m*
140.1432	131.0945	142.1589	131.0946	166.1591	131.0944	168.1746	131.0947	– H$_2$O - N(CH$_3$)$_3$
166.159	105.0787	168.1745	105.079	192.1741	105.0794	194.1902	105.0791	– N(CH$_3$)$_3$ - CO
184.1694	87.0683	186.1851	87.0684	210.1848	87.0687	212.2007	87.0686	- N(CH$_3$)$_3$ - CO
194.1539	77.0838	196.1694	77.0841	220.1649	77.0886	222.1851	77.0842	– H$_2$O - N(CH$_3$)$_3$
253.2271	18.0106	255.2427	18.0108	279.2428	18.0107	281.2583	18.0110	– H$_2$O

References
[1] K. Nakamura, Y. Tachikawa, M. Kitamura, O. Ohno, M. Suganuma and D. Uemura, *Org.Biomol. Chem.* **6**, 2058-2060 (2008); [2] R. Simonini, F. Maggioni, F. Zanetti, S. Fai, L. Forti, D. Prevedelli and S. Righi, *J. Exp. Mar. Biol. Ecol.* **534**, 151487 (2021); [3] S. Righi, L. Forti, R. Simonini, V. Ferrari, D. Prevedelli and A. Mucci, *Mar. Drugs* (2022), accepted.